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PROVISIONAL SPECIFICATION

for the invention entitled:

"A method of modulating ion channel functional activity"

The invention is described in the following statement:

- 1A -

A METHOD OF MODULATING ION CHANNEL FUNCTIONAL ACTIVITY

FIELD OF THE INVENTION

5 The present invention relates generally to a method of retarding, reducing or otherwise inhibiting membrane ion channel functional activity and, more particularly, Vpu ion channel functional activity. Even more particularly, the present invention provides a method of

treating HIV infection by inhibiting Vpu ion channel mediated HIV replication.

10 BACKGROUND OF THE INVENTION

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

15 Currently, no single treatment method is completely effective against HIV infections. Combination therapies, using drugs that target a number of different aspects of HIV replication, have proven to be the most effective way of ameliorating AIDS symptoms and prolonging life expectancy (1, 2, 3, 4, 5, 6, 7, 8, 9). For example, a measure of success has already been achieved with drugs targeting the viral reverse transcriptase and protease
20 enzymes (10, 11, 12, 13).

The protein Vpu forms an ion channel encoded by HIV and has a number of important roles in the virus life cycle including down-regulation of cell surface expression of the CD4 virus receptor molecule, control of the exit of gp160 from the endoplasmic reticulum and its
25 delivery to the cell surface and regulation of virion budding from the cell surface membrane. In the absence of Vpu, HIV replication has been shown to be severely retarded in monocytes and macrophages (14, 15).

To improve the prospect of treating and preventing HIV infection, there is an on-going need
30 to identify molecules capable of inhibiting various aspects of the HIV life cycle. In work leading up to the present invention, the inventors have surprisingly determined that although

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the drug amiloride has no effect of HIV replication, amiloride analogues in which the H₂N group located at the 5-position of the pyrazine has been substituted inhibit Vpu function thereby inhibiting the continuation of the HIV life cycle.

5 SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps
10 but not the exclusion of any other integer or step or group of integers or steps.

Accordingly, one aspect of the present invention provides a method of reducing, retarding or otherwise inhibiting membrane ion channel functional activity in a subject said method comprising administering to said subject an effective amount of an amiloride analogue or
15 functional equivalent thereof for a time and under conditions sufficient to inhibit membrane ion channel functional activity.

According to a preferred embodiment, the present invention provides a method of reducing, retarding or otherwise inhibiting Vpu ion channel mediation of HIV replication in a subject
20 said method comprising administering to said subject an effective amount of an amiloride analogue or functional equivalent thereof for a time and under conditions sufficient to inhibit Vpu ion channel mediation of HIV replication.

According to another preferred embodiment the present invention provides a method of
25 reducing, retarding or otherwise inhibiting Vpu ion channel functional activity in a subject said method comprising administering to said subject an effective amount of an amiloride analogue comprising a substitution of the amino group at the 5-position of the pyrazine ring or functional equivalent thereof for a time and under conditions sufficient to inhibit Vpu ion channel functional activity.

According to yet another preferred embodiment there is provided a method of reducing, retarding or otherwise inhibiting Vpu ion channel functional activity in a subject said method comprising administering to said subject an effective amount of HMA or functional equivalent thereof for a time and under conditions sufficient to inhibit Vpu ion channel functional
5 activity.

In another preferred embodiment there is provided a method of reducing, retarding or otherwise inhibiting Vpu ion channel functional activity in a subject said method comprising administering to said subject an effective amount of DMA or functional equivalent thereof for
10 a time and under conditions sufficient to inhibit Vpu ion channel functional activity.

Accordingly, in another aspect there is provided the method for the treatment or prophylaxis of HIV infection or AIDS in a subject said method comprising administering to said subject a Vpu ion channel functional activity inhibitory effective amount of an amiloride analogue or
15 functional equivalent thereof.

More particularly, the present invention provides a method for the treatment or prophylaxis of HIV infection or AIDS in a subject said method comprising administering to said subject an effective amount of an amiloride analogue or functional equivalent thereof wherein said
20 amiloride analogue reduces, retards or otherwise inhibits Vpu ion channel mediation of HIV replication.

The present invention further extends to the use of the subject amiloride analogues in the manufacture of a medicament for reducing, retarding or otherwise inhibiting ion channel
25 functional activity.

Yet another aspect of the present invention provides an agent useful for reducing, retarding or otherwise inhibiting membrane ion channel functional activity comprising an amiloride analogue or functional equivalent thereof as hereinbefore defined.

Another aspect of the present invention provides a composition for use in reducing, retarding or otherwise inhibiting membrane ion channel functional activity comprising an amiloride analogue or functional equivalent thereof as hereinbefore defined and one or more pharmaceutically acceptable carriers and/or diluents.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of plasmids used for expression of Vpu in *E. coli* **A**. The amino acid sequence (SEQ ID NO:1) encoded by the *vpu* open reading frame (ORF) generated by PCR from an HIV-1 strain HXB2 cDNA clone. The *vpu* ORF was cloned in-
10 frame at the 3' end of the GST gene in p2GEX to generate p2GEXVpu (**B**). It was subsequently cloned into pPL451 to produce the plasmid pPL+Vpu (**C**).

Figure 2 is a photographic representation of the expression and purification of Vpu in *E. coli*.
15 **A**. Western blotting after SDS-PAGE was used to detect expressed Vpu in *E. coli* extracts. Lanes 1-4 contain samples, at various stages of purity, of Vpu expressed from p2GEXVpu: lane 1, GST-Vpu fusion protein isolated by glutathione-agarose affinity chromatography; lane 2, Vpu liberated from the fusion protein by treatment with thrombin; lane 3, Vpu purified by HPLC anion exchange chromatography; lane 4, Vpu after passage through the
20 immunoaffinity column. Lanes 5 and 6, membrane vesicles prepared from 42°C induced cells containing pPL+Vpu or pPL451, respectively. **B**. Silver stained SDS-PAGE gel: lane 1, Vpu purified by HPLC anion exchange chromatography; lane 2, Vpu after passage through the immunoaffinity column.

25 **Figure 3** is a graphical representation of ion channel activity observed after exposure of lipid bilayers to aliquots containing purified Vpu. In **A** and **B**, the CIS chamber contained 500mM NaCl and the TRANS chamber contained 50mM NaCl; both solutions were buffered at pH 6.0 with 10 mM MES. **B** shows a current versus voltage curve generated from data similar to that shown in **A**.

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Figure 4 is a photographic representation of bacterial cross-feeding assays. Refer Methods section for a full description of this assay. For all plates, the Met⁻, Pro⁻ auxotrophic strain was used to seed a soft agar overlay. Plates **A** and **B** contain minimal drop-out medium minus proline; in plate **C** the medium was minus methionine. To control for viability of the cells in the background lawn, the discs labelled P and M contained added proline or methionine, respectively. The discs labelled C and V were inoculated with Met⁺, Pro⁺ *E. coli* cells containing the plasmids pPL451 or pPL+Vpu, respectively. Plates were incubated at 37°C (**A** and **C**) or 30°C (**B**) for two days and photographed above a black background with peripheral illumination from a fluorescent light located below the plate. The images were recorded on a Novaline video gel documentation system. Light halos around the discs labelled P or M on all plates and around the disc labelled V on plate **A** indicate growth of the background lawn strain.

Figure 5 is a graphical representation of the screening of drugs for potential Vpu channel blockers. The photograph shows a section of a minimal medium-lacking adenine - agarose plate onto which a lawn of XL-1-blue *E. coli* cells containing the Vpu expression plasmid pPLVpu has been seeded. Numbers 6-11 are located at the sites of application of various drugs being tested, which were applied in 3µl drops and allowed to soak into the agarose. The plate was then incubated at 37°C for 48hr prior to being photographed. The background grey shade corresponds to areas of no bacterial growth. The bright circular area around "10" represents bacterial cell growth as a result of application of adenine at that location (positive control). The smaller halo of bacterial growth around "9" is due to the application of 5-(N,N-hexamethylene)-amiloride at that location.

Figure 6 is a graphical representation of the inhibition of Vpu ion channel activity by 5-(N,N-hexamethylene)-amiloride (HMA) in planar lipid bilayers. The three traces represent typical Vpu channel activity observed in the presence of the indicated concentrations of HMA. The solid line indicates the zero current level. Mean currents (± variance), calculated for continuous channel recordings of at least 30 seconds duration, are plotted in the graph for each of the three drug concentrations.

Figure 7 is a graphical representation of the effect of HMA on HIV virion production in monocytes and monocyte-derived macrophages. HIV in culture supernatants was assayed at various days post-infection by detection of p24 antigen using a quantitative ELISA method. Solid black bars represent HIV-infected cells exposed to 10 μ M HMA. Hatched bars are control cells not exposed to drug.

Figure 8 is a schematic representation of the chemical formula of amiloride, HMA and DMA: R=H₂N, amiloride: R=(CH₃)₂N, DMA; R=(CH₂)₆N, HMA.

10 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated, in part, on the determination that although amiloride has no effect on HIV replication, amiloride analogues are able to inhibit the HIV life cycle by inhibiting Vpu ion channel functioning. This determination now permits the use of amiloride analogues as anti-viral agents for therapy and prophylaxis.

Accordingly, one aspect of the present invention provides a method of reducing, retarding or otherwise inhibiting membrane ion channel functional activity in a subject said method comprising administering to said subject an effective amount of an amiloride analogue or functional equivalent thereof for a time and under conditions sufficient to inhibit membrane ion channel functional activity.

Reference to "membrane ion channel" should be understood as a reference to a structure which transports ions across a membrane. The present invention extends to ion channels which may function by means such as passive, osmotic or active transport. The ion channel may be formed by intracellular or extracellular means. For example, the ion channel may be an ion channel which is naturally formed by a cell to facilitate its normal functioning. Alternatively, the ion channel may be formed by extracellular means. Extracellular means would include, for example, the formation of ion channels due to introduced chemicals, drugs or other agents such as ionophores or due to the functional activity of viral proteins encoded by a virus which has entered a cell. Preferably, the ion channel of the present invention is

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an ion channel which results from the infection of a cell with HIV and, more particularly, the ion channel is formed by the HIV protein Vpu (referred to herein as a "Vpu ion channel").

The ion channels which are the subject of the present invention facilitate the transport of ions
5 across membranes. Said membrane may be any membrane and is not limited to the outer cell wall plasma membrane. Accordingly, "membrane" encompasses the membrane surrounding
any cellular organelle, such as the Golgi apparatus and endoplasmic reticulum, the outer cell membrane, the membrane surrounding any foreign antigen which is located within the cell (for example, a viral envelope) or the membrane of a foreign organism which is located
10 extracellularly. The membrane is typically, but not necessarily, composed of a fluid lipid bilayer. The subject ion channel may be of any structure. For example, the Vpu ion channel is formed by Vpu which is an integral membrane protein encoded by HIV-1 which associates with, for example, the Golgi and endoplasmic reticulum membranes of infected cells. Reference hereinafter to "Vpu ion channels" should be read as including reference to all other
15 ion channels.

Accordingly, the present invention more particularly provides a method of reducing, retarding or otherwise inhibiting Vpu ion channel functional activity in a subject said method comprising administering to said subject an effective amount of an amiloride analogue or
20 functional equivalent thereof for a time and under conditions sufficient to inhibit Vpu ion channel functional activity.

Without limiting the present invention in any way, Vpu is a protein comprising approximately 80 amino acids with an N-terminal transmembrane anchor and a hydrophilic cytoplasmic C-
25 terminal domain. The C-terminal domain typically comprises a 12 amino acid sequence that is conserved and contains two serine residues which are phosphorylated (17, 18).

Reference to the "functional activity" of an ion channel should be understood as a reference to any one or more of the functions which an ion channel performs or is involved in. For
30 example, the Vpu protein encoded ion channel, in addition to facilitating the transportation

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of Na^+ , K^+ , Cl^- and PO_4^{3-} , also plays a role in the degradation of the CD4 molecule in the endoplasmic reticulum. The Vpu protein encoded ion channel is also thought to play a role in mediating the HIV life cycle since inactivating this channel inhibits the HIV life cycle, in particular, the replication of HIV. However, the present invention is not limited to treating
5 HIV infection via the mechanism of inhibiting the HIV life cycle and, in particular, HIV replication. ~~Rather, the present invention should be understood to encompass any mechanism~~
by which inhibiting Vpu ion channel functional activity acts to reduce, retard or otherwise inhibit HIV viability or functional activity. Said functional activity is preferably mediation of the replication of HIV.

10

According to this preferred embodiment, the present invention provides a method of reducing, retarding or otherwise inhibiting Vpu ion channel mediation of HIV replication in a subject said method comprising administering to said subject an effective amount of an amiloride analogue or functional equivalent thereof for a time and under conditions sufficient to inhibit
15 Vpu ion channel mediation of HIV replication.

Reference to "reducing, retarding or otherwise inhibiting" ion channel functional activity, and in particular Vpu mediation of HIV replication should be understood as a reference to inducing or facilitating the inhibition of said activity by both direct and indirect mechanisms.
20 For example, said amiloride analogue may interact directly with an ion channel such as a Vpu ion channel to prevent HIV replication or, alternatively may act indirectly to prevent said replication by, for example, interacting with a molecule other than the Vpu ion channel wherein said other molecule interacts with and inhibits the activity of the Vpu ion channel.

25 Reference to "HIV" should be understood as a reference to any HIV strains including homologues and mutants. Reference to the "HIV replication" should be understood as a reference to any one or more stages or aspects of the HIV life cycle, such as inhibiting the assembly or release of HIV virions. Said Vpu mediation of HIV replication may be by direct or indirect means. Said Vpu mediation is by direct means if the Vpu ion channel interacts
30 directly with HIV at any one or more of its life cycle stages. Said Vpu mediation is indirect

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if it acts on a molecule other than HIV which other molecule either directly or indirectly modulates any one or more aspects or stages of the HIV life cycle. Accordingly, the method of the present invention encompassess the mediation of HIV replication via the induction of a cascade of steps which lead to the mediation of any one or more aspects or stages of the
5 HIV life cycle.

Without limiting the present invention to any one theory or mode of action, amiloride analogues are thought to inhibit HIV viron release from cells by causing the Vpu ion channel to become blocked. This blocking is effected by substituted amiloride but not by
10 unsubstituted amiloride. Unsubstituted amiloride is a pyrazinoylguanidine bearing amino groups on the 3- and 5- positions and a chloro group on the 6- position of the pyrazine ring. However, the present invention should not be understood as limited to analogues of this form of amiloride. The present invention relates to analogues of any form of amiloride. For example, other isomeric forms of amiloride. Accordingly, reference to "amiloride analogue"
15 should be understood as a reference to any amiloride molecule which exhibits an addition, deletion or substitution, such as an addition, deletion or substitution of an atom or molecule or changing of the charge of an atom or molecule, at any position but more particularly at any one or more of the 6 positions of the pyrazine ring. Preferably, said amiloride analogue is an amiloride molecule exhibiting a substitution of the amino group at the 5-position of the
20 pyrazine ring.

According to this preferred embodiment the present invention provides a method of reducing, retarding or otherwise inhibiting Vpu ion channel functional activity in a subject said method comprising administering to said subject an effective amount of an amiloride analogue
25 comprising a substitution of the amino group at the 5-position of the pyrazine ring or functional equivalent thereof for a time and under conditions sufficient to inhibit Vpu ion channel functional activity.

Preferably, said functional activity is mediation of HIV replication.

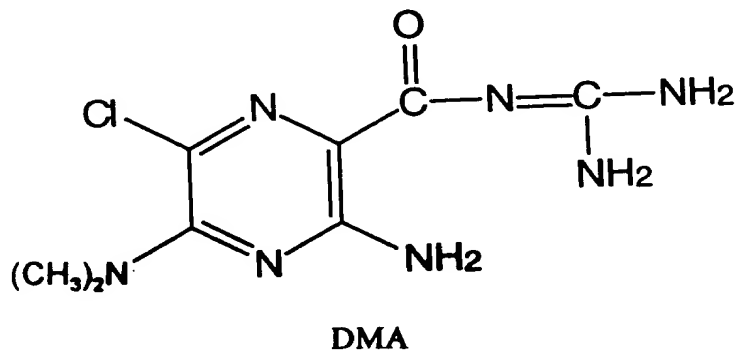
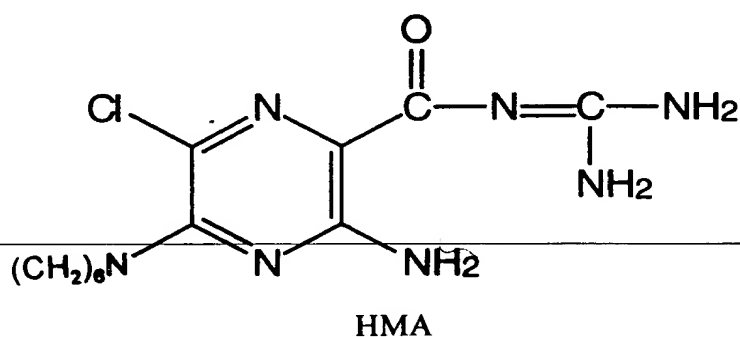
- 10 -

Still more preferably, said amiloride analogue is 5-(N,N-Hexamethylene)-Amiloride (referred to herein as "HMA") or 5-(N,N-Dimethyl)-Amiloride (referred to herein as "DMA").

According to this preferred embodiment there is provided a method of reducing, retarding or otherwise inhibiting Vpu ion channel functional mediation of HIV replication in a subject said method comprising administering to said subject an effective amount of HMA or functional equivalent thereof for a time and under conditions sufficient to inhibit Vpu ion channel mediation of HIV replication.

In another preferred embodiment there is provided a method of reducing, retarding or otherwise inhibiting Vpu ion channel functional mediation of HIV replication in a subject said method comprising administering to said subject an effective amount of DMA or functional equivalent thereof for a time and under conditions sufficient to inhibit Vpu ion channel mediation of HIV replication.

Most preferably said amiloride analogues comprise the structure:



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"Functional equivalents" of amiloride analogues include functionally active, fragments, parts, portions and chemical equivalents. Chemical equivalents may not necessarily be derived from an amiloride analogue but may share certain conformational similarity. Alternatively, chemical equivalents may be specifically designed to mimic certain physiochemical properties of the amiloride analogue. Chemical equivalents may be chemically synthesised or may be detected following, for example, natural product screenings. Functional equivalents may also possess antagonistic or agonistic properties and the use of such molecules are contemplated by the present invention.

10 The subject of the ion channel inhibition is generally an animal or bird such as but not limited to human, primate, livestock animal (e.g. sheep, cow, horse, donkey, pig), companion animal (e.g. dog, cat), laboratory test animal (e.g. mouse, rabbit, rat, guinea pig, hamster), captive wild animal (e.g. fox, deer), caged bird (e.g. parrot) and poultry bird (e.g. chicken, duck, pheasant, goose, turkey). Preferably, the subject is a human or primate. Most preferably, 15 the subject is a human.

The method of the present invention is useful in the treatment and prophylaxis of HIV infection and AIDS. For example, the amiloride analogues or functional equivalents of the present invention may be delivered into subjects known to be infected with HIV in order to 20 prevent replication of HIV thereby preventing the onset of AIDS. Alternatively, the method of the present invention may be used to reduce serum viral load or to alleviate AIDS symptoms.

The method of the present invention may be particularly useful either early in HIV infection 25 to prevent the establishment of a viral reservoir in cell types such as monocytes and macrophages or as a prophylactic treatment to be applied immediately prior to or for a period after exposure to a possible source of HIV infection.

Accordingly, in another aspect there is provided the method for the treatment or prophylaxis 30 of HIV infection or AIDS in a subject said method comprising administering to said subject

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a Vpu ion channel functional activity inhibitory effective amount of an amiloride analogue or functional equivalent thereof.

More particularly, the present invention provides a method for the treatment or prophylaxis
5 of HIV infection or AIDS in a subject said method comprising administering to said subject
an effective amount of an amiloride analogue or functional equivalent thereof wherein said
amiloride analogue reduces, retards or otherwise inhibits Vpu ion channel mediation of HIV
replication.

10 Preferably said amiloride analogue is HMA or DMA.

In accordance with this method, more than one type of amiloride analogue may be
administered or the amiloride analogue may be co-administered with a known anti-viral
compound or molecule. By "co-administered" is meant simultaneous administration in the
15 same formulation or in two different formulations via the same or different routes or
sequential administration by the same or different routes. By "sequential" administration is
meant a time difference of from seconds, minutes, hours or days between the administration
of the two types of an amiloride analogue or the amiloride analogue and the known anti-viral
compound or molecule. The amiloride analogue or the amiloride analogue and known anti-
20 viral compound or molecule may be administered in any order.

Routes of administration includes but are not limited to intravenously, intraperitoneal,
subcutaneously, intracranial, intradermal, intramuscular, intraocular, intrathecal,
intracerebrally, intranasally, infusion, orally, rectally, *via* iv drip, patch and implant.
25 Intravenous routes are particularly preferred.

The present invention further extends to the use of the subject amiloride analogues in the
manufacture of a medicament for reducing, retarding or otherwise inhibiting ion channel
functional activity.

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Preferably said ion channel is a Vpu ion channel and even more preferably said functional activity is mediation of HIV replication.

Most preferably said amiloride analogues are HMA or DMA.

5

Yet another aspect of the present invention provides an agent useful for reducing, retarding or otherwise inhibiting membrane ion channel functional activity comprising an amiloride analogue or functional equivalent thereof as hereinbefore defined.

10 Preferably said ion channel is a Vpu ion channel and even more preferably said functional activity is mediation of HIV replication.

Most preferably said amiloride analogues are HMA or DMA.

15 Another aspect of the present invention provides a composition for use in reducing, retarding or otherwise inhibiting membrane ion channel functional activity comprising an amiloride analogue or functional equivalent thereof as hereinbefore defined and one or more pharmaceutically acceptable carriers and/or diluents. The composition may also comprise two difference types of amiloride analogues or an amiloride analogue and a known anti-viral
20 compound or molecule.

Preferably said inhibition of ion channel functional activity is inhibition of Vpu ion channel mediation of HIV replication.

25 Compositions suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions. They must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for
30 example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable

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mixtures thereof and vegetable oils. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged
5 absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as
10 required, followed by, for example, filter sterilization or sterilization by other appropriate means. Dispersions are also contemplated and these may be prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, a preferred method of
15 preparation includes vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution.

When the active ingredients are suitably protected, they may be orally administered, for
20 example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of
25 active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1mg and
30 2000 mg of active compound.

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The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: A binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or
5 saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain
10 the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

15

The present invention also extends to forms suitable for topical application such as creams, lotions and gels. In such forms, the anti-clotting peptides may need to be modified to permit penetration of the surface barrier.

20 Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary
25 active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated;
30 each unit containing a predetermined quantity of active material calculated to produce the

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desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved and (b) the limitations inherent in the art of compounding such an active
5 material for induce or facilitating analgesia in living subjects.

Effective amounts of anti-clotting tannins contemplated by the present invention will vary depending on the severity of the pain and the health and age of the recipient. In general terms, effective amounts may vary from 0.01 ng/kg body weight to about 100 mg/kg body
10 weight. Alternative amounts include for about 0.1 ng/kg body weight about 100 mg/kg body weight or from 1.0 ng/kg body weight to about 80 mg/kg body weight.

Further features of the present invention are more fully described in the following Examples. It is to be understood, however, that the detailed description is included solely for the purpose
15 of exemplifying the present invention. It should not be understood in any way as a restriction on the broad description of the invention as set out above.

EXAMPLE 1

Construction of recombinant plasmids p2GEXVpu and pPLVpu

The open reading frame encoding Vpu (Fig 1a) was amplified by PCR from a cDNA clone
5 of an Nde 1 fragment of the HIV-1 genome (isolate HXB2, a gift from Dr N. Deacon,
McFarlane Burnet Centre, Melbourne, Australia). Native *Pfu* DNA polymerase (Stratagene;
0.035 U/ μ l) was chosen to catalyse the PCR reaction to minimise possible PCR introduced
errors by virtue of the enzyme's proofreading activity. The 5', sense, primer
AGTAGGATCCATGCAACCTATACC (SEQ ID NO:2) introduces a BamH1 site
10 (underlined) for cloning in-frame with the 3' end of the GST gene in p2GEX (41). This
primer also repairs the start codon (bold T replaces a C) of the *vpu* gene which is a threonine
codon in the HXB2 isolate. The 3', antisense, primer TCTGGAATTCTACAGATCAT
CAAC (SEQ ID NO:3) introduces an EcoR1 site (underlined) to the other end of the PCR
product to facilitate cloning. After 30 cycles of 94°C for 45 sec, 55°C for 1 min and 72°C
15 for 1 min in 0.5 ml thin-walled eppendorf tubes in a Perkin-Elmer thermocycler, the 268bp
fragment was purified, digested with BamH1 and EcoR1 and ligated to p2GEX prepared by
digestion with the same two enzymes. The resultant recombinant plasmid is illustrated in Fig
1b. The entire Vpu open reading frame and the BamH1 and EcoR1 ligation sites were
sequenced by cycle sequencing, using the Applied Biosystems dye-terminator kit, to confirm
20 the DNA sequence.

To prepare the Vpu open reading frame for insertion into the pPL451 expression plasmid,
p2GEXVpu was first digested with BamH1 and the 5' base overhang was filled in the Klenow
DNA polymerase in the presence of dNTPs. The Vpu-encoding fragment was then liberated
25 by digestion with EcoR1, purified from an agarose gel and ligated into pPL451 which had
been digested with Hpa1 and EcoR1. Western blots subsequently confirmed that the pPLVpu
construct (Fig 1c) expressed Vpu after induction of cultures at 42°C to inactivate the cI857
repressor of the PR and PL promoters.

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EXAMPLE 2

Raising polyclonal antibodies for immuno-identification of Vpu

A peptide CALVEMGVEMGGHHAPWDVDDL (SEQ ID NO:4) corresponding to the C-terminal 20 amino acid residues of Vpu was synthesised in the Biomolecular Resource Facility (ANU, Australia) using an Applied Biosystems model 477A machine. A multiple antigenic peptide (MAP) was prepared (23) by coupling the peptide to a polylysine core via the N-terminal cysteine residue. The MAP was used to immunise rabbits for production of polyclonal antisera recognising the C-terminus of Vpu. For immunisations 1mg of MAP peptide was dissolved in 1.25ml of MTPBS (16mM Na₂HPO₄, 4mM NaHPO₄, 150mM NaCl pH 7.3) and emulsified with 1.25ml of Freund's complete adjuvant and injected at multiple subcutaneous sites on the rabbit's back. Booster injections used Freund's incomplete adjuvant and were spaced at least 4 weeks apart with serum being sampled 10-14 days after injections.

EXAMPLE 3

Techniques involving the antibodies

Peptide-specific antibodies were purified from rabbit sera using an Immunopure™ Ag/Ab Immobilisation kit from Pierce. The synthetic peptide was cross-linked via its N-terminal cysteine to the matrix of a 5ml Sulfo Link™ column according to the kit's instructions, 2.5ml of Vpu immunoreactive serum was added to 20ml of Tris buffer (10mM pH 7.4) and passed through the peptide column three times to maximise exposure of the antibodies to the peptide. The column was washed with 20ml of 10mM Tris pH 7.4 followed by 20ml of the same buffer supplemented with 500mM NaCl. The bound antibodies were eluted in 5ml of 100mM glycine/150mM NaCl, pH 2.5 and eluents were immediately neutralised by addition of 250μl of 1M Tris pH 9.0 and dialysed overnight against MTPBS.

An anti-Vpu immunoaffinity column was constructed by covalently cross-linking 200μg of purified antibody to 100μl of protein A agarose beads (Schleicher and Schuell) using the bifunctional cross-linking reagent dimethylpylimelidate as described previously (24).

Immunoprecipitation of Vpu was performed by incubation of samples in the presence of approximately 5-fold excess of purified antibody (room temperature for 1 hr) followed by addition of excess protein-A agarose, incubation for 30 min, and centrifugation to pellet the Vpu-antibody complexes. The supernatant, which was subsequently used as a control in the
5 electrophysiological bilayer experiments, was tested by western blotting to confirm that Vpu had been completely removed. Protein samples were electrophoresed on homogeneous 18% SDS polyacrylamide gels using a minigel apparatus and prepoured gels (Novex). Samples were treated with SDS (3.2% final) and mercaptoethanol (0.8% final) at 60°C for 5 min before loading onto gels. Protein bands were visualised either with Coomassie brilliant blue
10 R250 or by silver staining.

For western blotting, proteins were transferred from acrylamide gels to PVDF membranes using a semi-dry transfer apparatus (Pharmacia LKB). Vpu was detected after consecutive reactions of the blots with polyclonal antiserum or purified antibodies, goat anti-rabbit
15 alkaline phosphatase conjugate and Western Blue™ stabilised substrate (Promega).

Example 4

Purification of recombinant Vpu from *E. coli*

20 Cultures of *E. coli* strain XL1-blue cells containing p2GEXVpu were grown at 30°C with vigorous aeration in LB medium supplemented with glucose (6g/L) and ampicillin (50mg/L) to a density of approximately 250 Klett units, at which time IPTG was added to a final concentration of 0.01mM and growth was continued for a further 4hr. The final culture density was approximately 280 Klett units. Since early experiments revealed that the majority
25 of expressed GST-Vpu fusion protein was associated with both the cell debris and membrane fractions, we adopted the method of Varadhachary and Maloney (25) to isolate osmotically disrupted cell ghosts (combining both cell debris and membrane fractions) for the initial purification steps. Cells were harvested, washed, weighed and resuspended to 10ml/g wet weight in MTPBS containing DTT (1mM) and MgCl₂ (10mM). Lysozyme (0.3 mg/ml;
30 chicken egg white; Sigma) was added and incubated on ice for 30 min with gentle agitation

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followed by 5 min at 37°C. The osmotically sensitised cells were pelleted at 12,000g and resuspended to the original volume in water to burst the cells. The suspension was then made up to 1xMTPBS/DTT using a 10x buffer stock and the ghosts were isolated by centrifugation and resuspended in MTPBS/DTT to which was then sequentially added glycerol (to 20%
5 wt/vol) and CHAPS (to 2% wt/vol) to give a final volume of one quarter the original volume. This mixture was stirred on ice for 1 hr and then centrifuged at 400,000g for 1hr to remove insoluble material. The GST-Vpu fusion protein was purified from the detergent extract by affinity chromatography on a glutathione agarose resin (Sigma). The resin was thoroughly washed in 50mM Tris pH 7.5 containing glycerol (5%), DTT (1mM), and CHAPS (0.5%)
10 (Buffer A) and then the Vpu portion of the fusion protein was liberated and eluted from the resin-bound GST by treatment of a 50% (v/v) suspension of the beads with human thrombin (100U/ml; 37°C for 1hr). PMSF (0.5mM) was added to the eluant to eliminate any remaining thrombin activity. This Vpu fraction was further purified on a column of MA7Q anion exchange resin attached to a BioRad HPLC and eluted with a linear NaCl gradient (0-
15 2M) in buffer A.

The Vpu was purified to homogeneity - as determined on silver stained gels - on an immunoaffinity column as follows: HPLC fractions containing Vpu were desalted on a NAP 25 column (Pharmacia) into buffer A and then mixed with the antibody-agarose beads for 1hr
20 at room temperature. The beads were washed thoroughly and Vpu was eluted by increasing the salt concentration to 2M. Protein was quantitated using the BioRad dye-binding assay.

Example 5

Reconstitution of Vpu in phospholipid vesicles

25

Proteoliposomes containing Vpu were prepared by the detergent dilution method (45). A mixture of lipids (PE:PC:PS; 5:3:2; 1mg total lipid) dissolved in chloroform was dried under a stream of nitrogen gas and resuspended in 0.1 ml of potassium phosphate buffer (50mM pH 7.4) containing DTT (1mM). A 25 μ l aliquot containing purified Vpu was added, followed
30 by octylglucoside to a final concentration of 1.25% (wt/vol). This mixture was subject to

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three rounds of freezing in liquid nitrogen, thawing and sonication in a bath-type sonicator (20-30 sec) and was then rapidly diluted into 200 volumes of the potassium phosphate buffer. Proteoliposomes were collected by centrifugation at 400,000g for 1hr and resuspended in approximately 150 μ l of phosphate buffer.

5

Example 6

Assaying ion channel activity

Purified Vpu was tested for its ability to induce channel activity in planar lipid bilayers using
10 standard techniques as described elsewhere (27; 22). The solutions in the CIS and TRANS
chambers were separated by a DelrinTM plastic wall containing a small circular hole of
approximately 100 μ m, diameter across which a lipid bilayer was painted so as to form a high
resistance electrical seal. Bilayers were painted from a mixture (8:2) of palmitoyl-oleoly-
phosphatidyl-ethanolamine and palmitoyl-oleoly-phosphatidyl-choline (Avanti Polar Lipids,
15 Alabaster, Alabama) in n-decane. The solutions in the two chambers contained MES buffer
(10mM, pH 6.0) to which various NaCl or KCl concentrations were added. Currents were
recorded with an AxopatchTM 200 amplifier. The electrical potential between the two
chambers could be manipulated between \pm 200mV (TRANS relative to grounded CIS).
Aliquots containing Vpu were added to the CIS chamber either as a detergent solution or after
20 incorporation of the protein into phospholipid vesicles. The chamber was stirred until
currents were observed.

Example 7

Testing the effect of HMA and DMA on HIV replication in human monocytes and macrophages

25

In tests performed at The Centre for Virus Research (Westmead Hospital, Sydney, Australia),
human monocytes were isolated from peripheral blood and cultured either for 24hr (one day
old monocytes) or for 7 days to allow differentiation into monocyte derived macrophages
30 (MDM). These cells were then exposed to cell-free preparations of HIV isolates and allowed

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to absorb for 2hr before complete aspiration of the medium, washing once with virus-free medium and resuspension in fresh medium. The cells were exposed to 50-10 μ m HMA or DMA either 24 hr prior to infection or after infection. Subsequent HIV replication, at various times after infection, was compared in cells exposed to drugs and in cells not exposed to
5 drugs (controls). The progression and extent of viral replication was assayed using either an HIV DNA PCR method (28) or an ELISA method to quantitate p24 in culture supernatants
(29).

Example 8

10 Expression and purification of Vpu in E. Coli

The plasmid p2GEXVpu (Fig. 1) was constructed to create an in-frame gene fusion between the GST and Vpu open-reading frames. This system enabled IPTG-inducible expression of the Vpu polypeptide fused to the C-terminus of GST and allowed purification of the fusion
15 protein by affinity chromatography on glutathione agarose.

Optimal levels of GST-Vpu expression were obtained by growing the cultures at 30°C to a cell density of approximately 250-300 Klett units and inducing with low levels of IPTG (0.01mM). To purify the GST-Vpu, a combined cellular fraction containing the cell debris
20 and plasma membrane was prepared by lysozyme treatment of the induced cells followed by a low-speed centrifugation. Approximately 50% of the GST-Vpu protein could be solubilised from this fraction using the zwitterionic detergent CHAPS. Affinity chromatography using glutathione-agarose beads was used to enrich the fusion protein and thrombin was used to cleave the fusion protein at the high affinity thrombin site between the fusion partners,
25 liberating Vpu (Fig. 2A). In fractions eluted from the anion exchange column Vpu was the major protein visible on silver stained gels (Fig. 2B, lane 1). Finally, Vpu was purified to apparent homogeneity on an immunoaffinity column (Fig. 2B, lane 2). The N-terminal amino acid sequence of the protein band (excised from SDS-PAGE gels) corresponding to the immunodetected protein confirmed its identity as Vpu.

Example 9

Vpu forms ion channels in lipid bilayers

To assay for ion-channel formation by Vpu, reconstitution into planar lipid bilayers was performed. When samples (containing between 7 and 70ng of protein) of purified recombinant Vpu were added to the 1ml of buffer in the CIS chamber of the bilayer apparatus, current fluctuations were detected after periods of stirring that varied from 2 to 30 min (Fig. 3). This time taken to observe channel activity approximately correlated with the amount of protein added to the chamber. No channels were detected when control buffer aliquots or control lipid vesicles were added to the CIS chamber. In those control experiments the chambers could be stirred for more than an hour without appearance of channel activity.

Example 10

Properties of the Vpu channels

Channel activity was observed in over 40 individual experiments with Vpu samples prepared from five independent purifications. In different experiments, the amplitude of the currents varied over a large range and, again, seemed to approximately correlate with the amount of protein added. The smallest and largest channels measured had conductances of 14 pS and 280 pS, respectively. The channels were consistently smaller when lipid vesicles containing Vpu were prepared and fused to the bilayer rather than when purified protein in detergent solution was added. This may be because the former method included treatment with high concentrations of detergent and a dilution step that may have favoured the breakdown of large aggregates into monomers.

The relationship between current amplitude and voltage was linear and the reversal potential in solutions containing a ten-fold gradient of NaCl (500mM CIS; 50mM TRANS) was +30mV (Fig. 3B). A similar reversal potential was obtained when solutions contained KCl instead of NaCl. In 5 experiments with either NaCl or KCl in the solutions on either side of

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the membrane, the average reversal potential was $31.0 \pm 1.2\text{mV}$ ($\pm\text{SEM}$). This is more negative than expected for a channel selectively permeable for the cations alone. Using ion activities in the Goldman-Hodgkin-Katz equation gives a $P_{\text{Na}}/P_{\text{Cl}}$ ratio of about 5.5 indicating that the channels are also permeable to chloride ions. An attempt was made to reduce the anion current by substituting phosphate for chloride ions. When a Na-phosphate gradient (150mM Na^+ & 100mM phosphate CIS; 15mM Na^+ & 10mM phosphate TRANS, pH 6.8) was used instead of the Na Cl gradient, the reversal potential was 37.1 ± 0.2 ($\pm\text{SEM}$, $n=2$) again indicating a cation/anion permeability ratio of about 5. (For calculations involving the phosphate solutions, the summed activities of the mono and bivalent anions were used and it was assumed that the two species were equally permeable). The current-voltage curve now exhibited rectification that was not seen in the NaCl solutions: We have no explanation for this difference. It can be concluded that the channels formed by Vpu are equally permeable to Na^+ and K^+ and are also permeable, though to a lesser extent, to chloride as well as phosphate ions.

15

Example 11

Bio-assay for screening potential ion-channel blocking drugs

As part of a search for drugs that block the Vpu ion channel, a novel bio-assay was developed by us to facilitate the screening process which would be prohibitively slow if performed in the bilayer assay (16). This bio-assay is based on the observation that expression of Vpu in *E.coli* results in an active Vpu channel located in the plasmalemma that dissipates the transmembrane sodium gradient. As a consequence of this Vpu channel activity, metabolites whose accumulation within the cells is mediated by a sodium dependent co-transporter (for example proline or adenine) leak out of the cell faster than they can be synthesised so that the metabolites' intracellular levels become limiting for growth of the cell. Thereby, and *E.coli* cell expressing Vpu is unable to grow in minimal drop-out media lacking adenine or proline. However, in the presence of a drug that blocks the Vpu channel, the cell is once again able to re-establish its transmembrane sodium gradient - due to the action of other ion pumps in the membrane - and the leakage of metabolites is prevented enabling growth. Experiments to

30

demonstrate that Vpu can form sodium channels in the plasma membrane of *E.coli* were performed as follows:

To express unfused Vpu in *E.coli*, the vpu open-reading frame was cloned into the plasmid pPL451 (19) to create the recombinant plasmid pPL-Vpu (Fig. 1b). In this vector the strong P_L and P_R lambda promoters are used to drive expression of Vpu under control of the temperature sensitive cI857 repressor, such that when grown at 30°C expression is tightly repressed and can be induced by raising the temperature to between 37°C and 42°C. On agar plates, cells containing pPL-Vpu grew when incubated at 30°C and 37°C but not at 42°C, while control strains grew well at 42°C. Liquid cultures of cells containing pPL-Vpu were grown at 30°C to $OD_{600}=0.84$ then moved to grow at 42°C for two hours (the final cell density was $OD_{600}=0.75$). The plasma membrane fraction was prepared and western blotting, using an antibody that specifically binds to the C-terminus of Vpu, detected a single band at approximately 16kDa, indicating that Vpu was expressed and associated with the membranes (Fig. 2A, lane 5).

Example 12

Cross-feeding experiments reveal that proline leaks out of cells expressing Vpu

Uptake of proline by *E.coli* is well characterised and active transport of the amino acid into the cells is known to use the sodium gradient as the energy source (20). We predicted that if the sodium gradient were dissipated by a sodium channel in the plasma membrane then proline synthesised in the cytoplasm will diffuse out of the cells. To detect whether this proline leakage occurred, the following cross-feeding assay was used: A lawn of an *E.coli* strain auxotrophic for proline and methionine (Met⁻ Pro⁻), was seeded and poured as a soft agar overlay on minimal drop-out media plates lacking proline but containing methionine. Sterile porous filter discs were inoculated with a Met⁺ Pro⁺ strain (XL-1 blue) containing either the pPL451 control plasmid or pPL-Vpu and placed onto the soft agar. The plates were then incubated at 37°C or 30°C for two days. After than time a halo growth of the Met⁻ Pro⁻ strain was clearly visible surrounding the disc

inoculated with the cells containing pPL-Vpu incubated at 37°C (Fig. 4A). This growth can only be due to the leakage of proline from the Vpu-expressing cells on the disc. No such leakage was apparent from the control strain at 37°C nor around either strain on plates grown at 30°C (Fig. 4B).

5

In contrast to proline transport, the *E.coli* methionine permease is known to belong to the ABC transporter family (21) and hence be energised by ATP. Identical cross-feeding experiments to those described above were set up except that the Met⁻ Pro⁻ strain was spread on minimal drop-out plates lacking methionine but containing proline. No growth of this strain was evident around any of the discs (Fig. 4C), indicating that methionine was not leaking out of the XL-1 blue cells even when Vpu was being expressed.

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Example 13

***E. Coli* cells expressing Vpu require adenine in the external medium for growth**

15

It was observed that, due to an uncharacterised mutation in the adenine synthesis pathway, growth of *E.coli* cells of the XL1-blue strain expressing Vpu at 37°C was dependant on the presence of adenine in the medium. This allowed the development of an even simpler bio-assay for Vpu ion-channel activity than the proline cross-feeding assay described above: A lawn of XL1-blue cells containing the pPL-Vpu plasmid is seeded onto an agarose plate lacking adenine in the medium, small aliquots of drugs to be tested for inhibition of the Vpu channel are spotted onto the agarose in discreet locations and the plates are incubated at 37°C for a suitable period of time (12-36 hours). Halos of growth around a particular drug application site indicate that the drug has inhibited expression of the Vpu ion channel activity that prevents growth in the absence of the drug.

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Example 14

The bioassay reveals 5-(N,N-Hexamethylene)-Amiloride as a potential channel blocker

5 Using this assay, a number of amantadine derivatives were tested but found not to affect channel activity. However, when a number of amiloride derivatives were also tested, a halo of growth around the site of application of 5-(N,N-Hexamethylene)-Amiloride (HMA) identified this drug as a potential Vpu channel blocker (Fig. 5). Unsubstituted amiloride did not produce a halo of bacterial growth on these plates.

10

Example 15

Planar lipid bilayer experiments confirm HMA as a Vpu channel inhibitor

Inhibition of the Vpu ion-channel activity by HMA was confirmed in planar lipid bilayer experiments (Fig. 6), where concentrations of 50-250 μ M HMA were found to block ion flow through the channel. The parent compound, amiloride, and another derivative, 5-(N,N-Dimethyl)-Amiloride (DMA), were similarly tested in planar lipid bilayer experiments: DMA was found to inhibit channel activity, though not as potently as HMA. Amiloride itself was not active as a channel blocker at these concentrations.

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Example 16

HMA and DMA inhibit HIV-1 replication in human monocytes and macrophages

Subsequent tests were carried out to establish whether there was any anti-viral activity of HMA and DMA. These tests were outsourced to The Centre for Virus Research, Westmead Hospital and performed by Prof. T. Cunningham and Dr H. Naif. Two tests were performed to characterise the effects of the drugs on HIV replication in human monocytes and macrophages: a) A PCR based assay was used to detect newly synthesised DNA arising from reverse transcription of the HIV genome, an early stage in virus replication; b) An ELISA method was used to quantitate production of the viral protein p24, reflecting a later stage in

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the replication process. Results of the PCR assay (data now shown) indicated that DMA at 50 μ M inhibited synthesis of HIV DNA in the cells; HMA was toxic to the cells at 50 μ M - further tests are being carried out at lower concentrations of this drug. p24 ELISA results indicated a clear inhibition of HIV virion synthesis with both DMA (50 μ M, data not shown) and also with HMA (Fig. 7), when used at non-toxic levels (10 μ M); Fig. 7A shows the effect of HMA on monocytes, Fig. 7B shows the effect of HMA on macrophages.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: ANUTECH PTY LIMITED
- (ii) TITLE OF INVENTION: A METHOD OF MODULATING ION CHANNEL
FUNCTIONAL ACTIVITY
-

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

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(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(C) TELEX: AA 31787

- 33 -

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 82 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Ile	Ile	Ala	Ile	Val	Val	Trp	Ser	Ile	Val	Ile	Ile	Glu	Tyr	Arg	Lys
			20					25					30		
Ile	Leu	Arg	Gln	Arg	Lys	Ile	Asp	Arg	Leu	Ile	Asp	Arg	Leu	Ile	Glu
			35				40					45			
Arg	Ala	Glu	Asp	Ser	Gly	Asn	Glu	Ser	Glu	Gly	Glu	Ile	Ser	Ala	Leu
		50				55					60				
Val	Glu	Met	Gly	Val	Glu	Met	Gly	His	His	Ala	Pro	Trp	Asp	Val	Asp
65					70					75				80	

Asp Leu

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
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(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGTAGGATCC ATGCAACCTA TACC

24

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
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 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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24

- 34 -

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
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 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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1				5				10						15	
Asp Val Asp Asp Leu															
20															

DATED this 12th day of October 1998

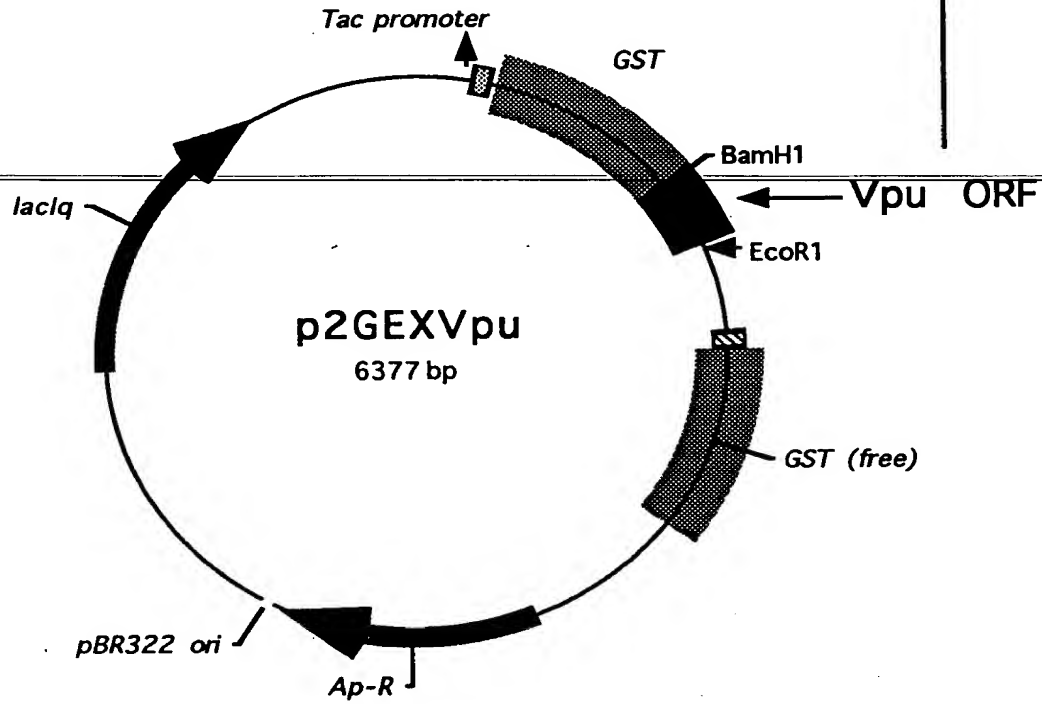
~~Anutech Pty Limited~~ The Australian Capital University
by their Patent Attorneys
DAVIES COLLISON CAVE



A.

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B.



C.

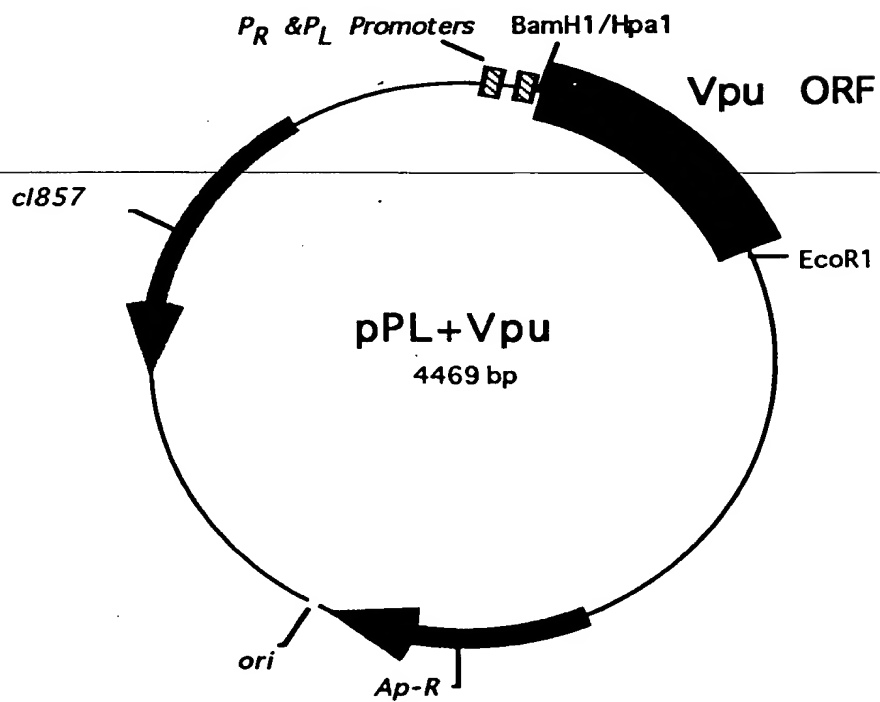


Figure 1

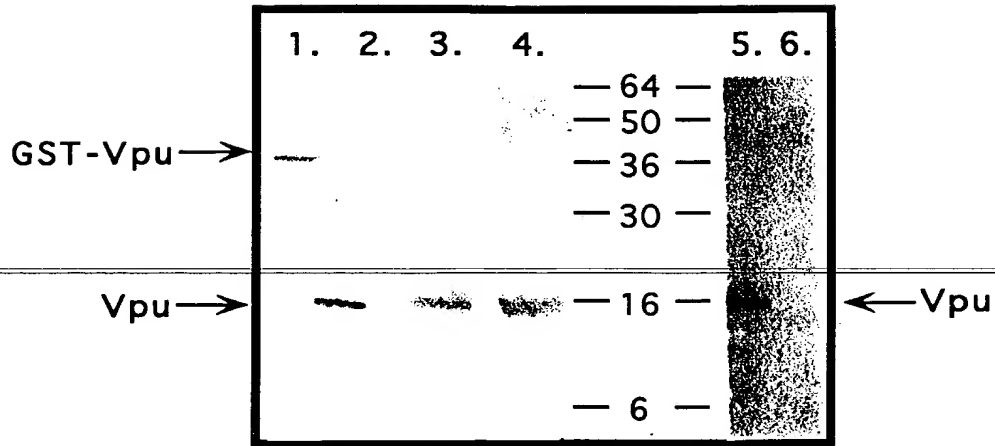
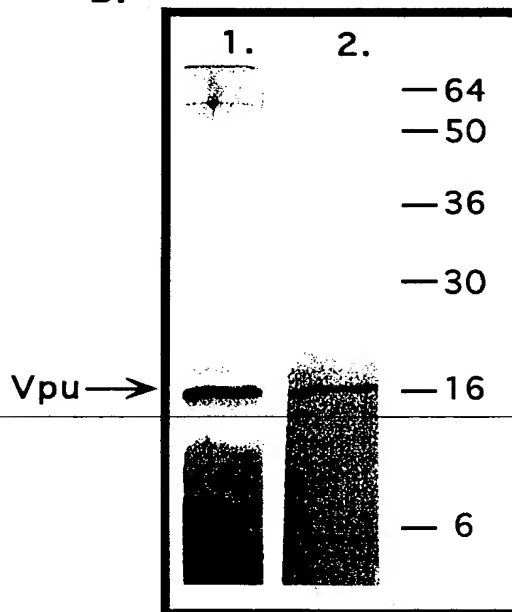
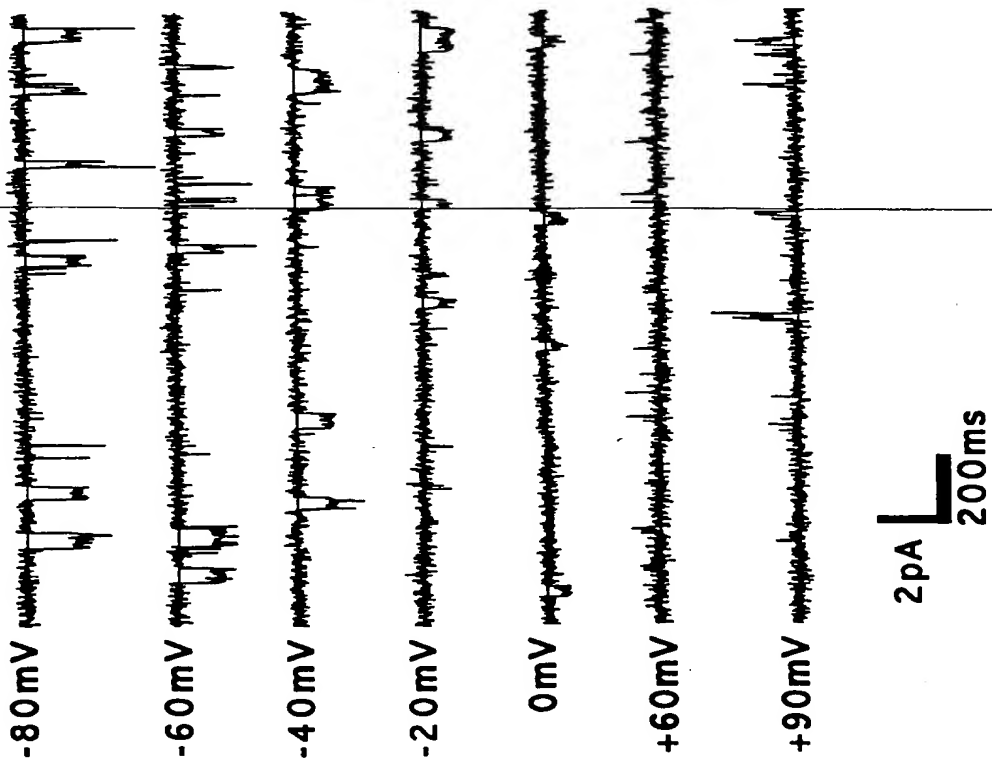
A. Western Blot**B. Silver Stained Gel**

Figure 2

B.



A.

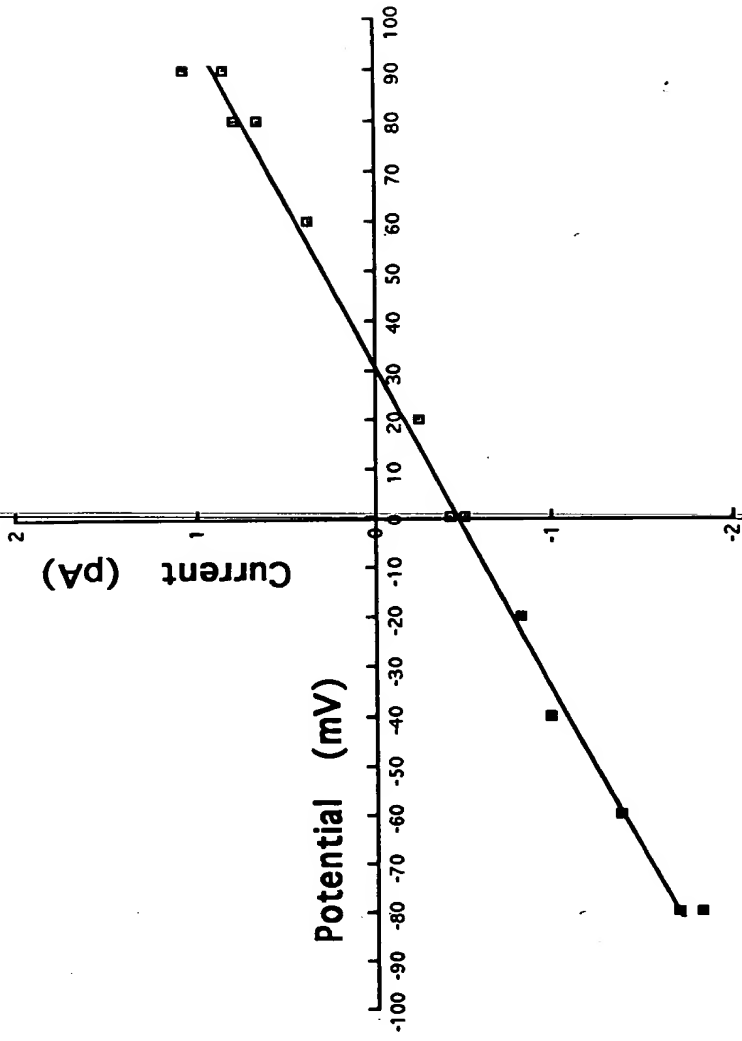
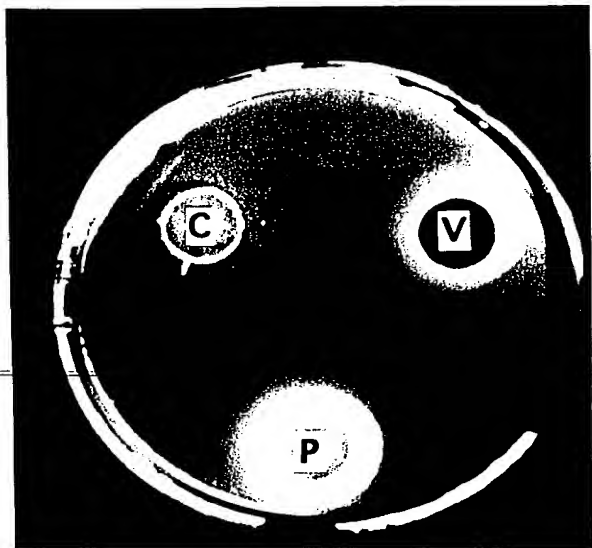
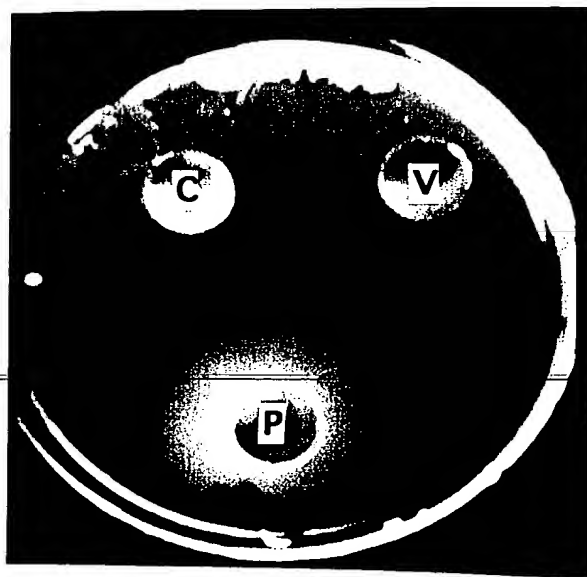


Figure 3

A. 37°C, Pro⁻ plate



B. 30°C, Pro⁻ plate



C. 37°C Met⁻ plate

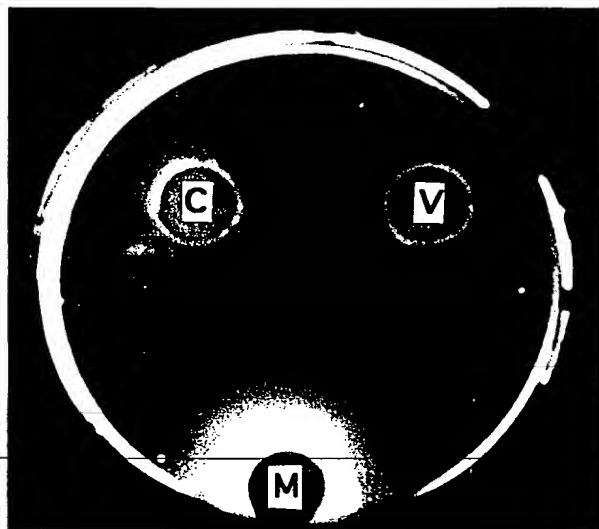


Figure 4

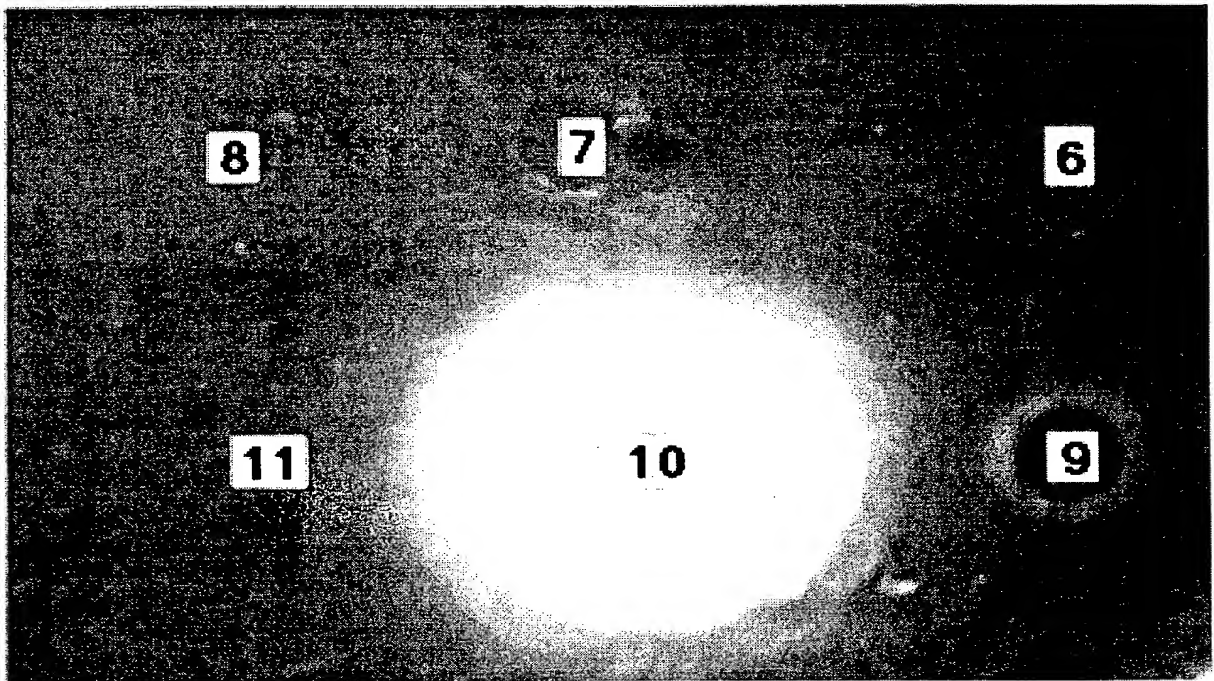


Figure 5

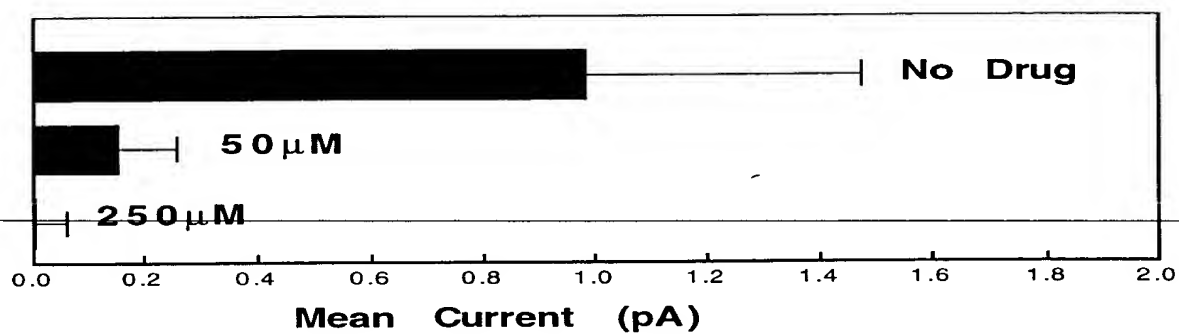
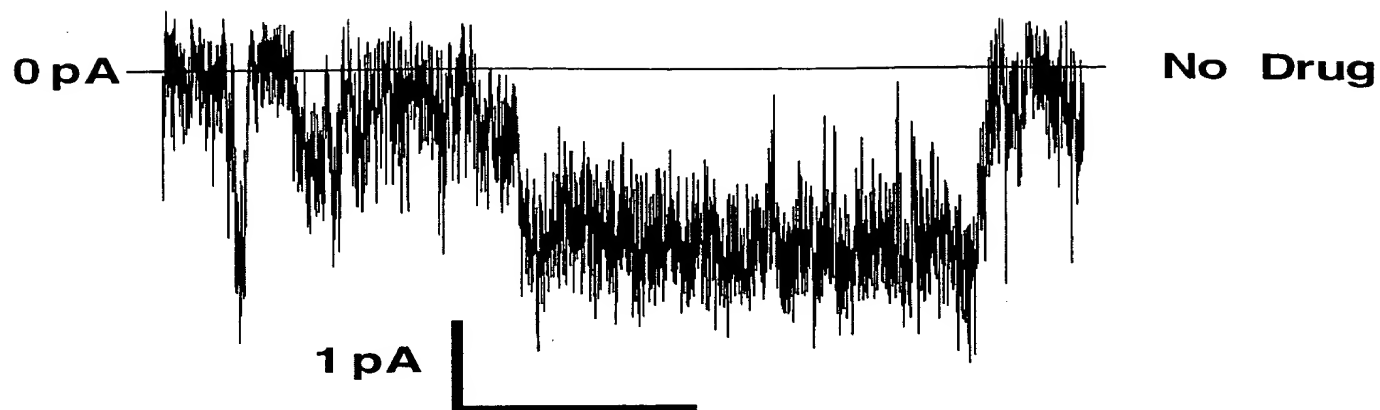


Figure 6

Inhibition of HIV replication by HMA

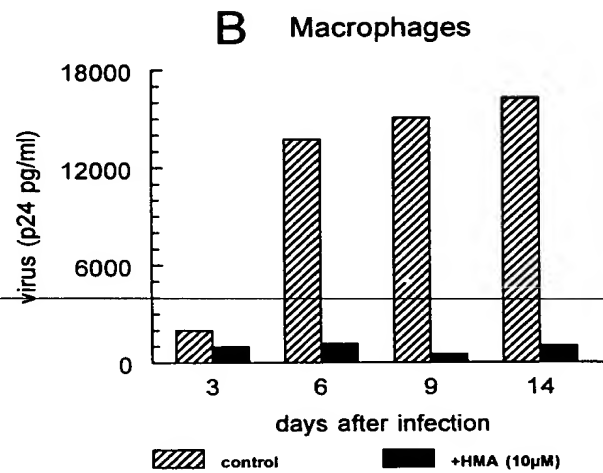
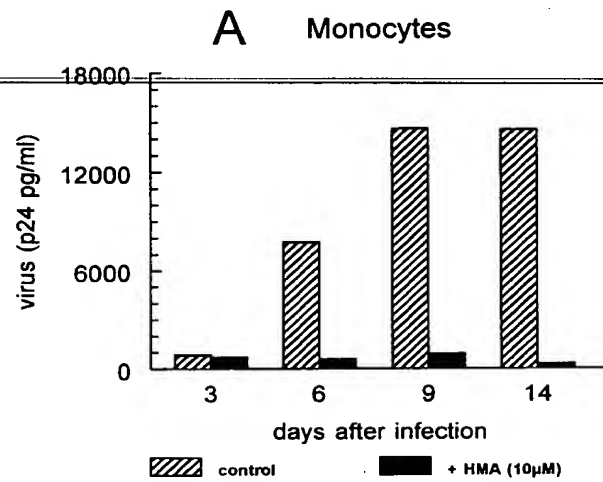


Figure 7

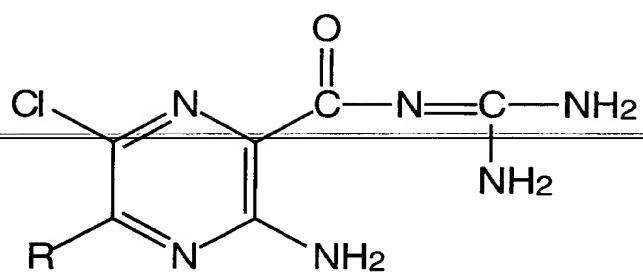


Figure 8

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